Journal of Forensic Medicine

P.O. Box 1010 · Johannesburg · South Africa

Sponsored by the South African Medico-Legal Society

Editor: H. A. Shapiro, Ph.D., M.B., Ch.B., F.R.S.S.Af.
Associate Editor: I. Gordon, M.B., Ch.B., F.R.S.S.Af.

Guest Editorial

Further Complexities of the Rh–Hr Blood Types

The paper by Maurice Shapiro[1] in this issue is a magnificent and fundamental contribution to the knowledge and understanding of the serology and genetics of the Rh–Hr types, with important implications, especially in cases of disputed parentage. Shapiro has shown that, reciprocally related to blood factor **rh″**, there exists in addition to the "standard" blood factor **hr″**, a related blood factor **hr^S**.* Thus, agglutinogens which have as one of their serologic attributes the blood factor **hr″**, are of two kinds: one (the common type) also has blood factor **hr^S**; while the other (extremely rare in Caucasians but not so rare in the Bantu) lacks the blood factor **hr^S**.

Blood factor **hr^S** bears a relationship to factor **hr″** analogous to the relationship between the recently discovered factors **Rh^A**, **Rh^B**, **Rh^C**, and **Rh^D** and blood factor **Rh$_0$**. The serological difference between blood factors **hr″** and **hr^S** is just as sharp as the difference between the factors **Rh$_0$**, **rh′**, **rh″**, **hr′** and **hr″**. By family studies Shapiro has shown that the newly defined Rh–Hr agglutinogens characterized by the absence of the expected factor **hr^S**, are inherited by corresponding allelic genes. To date Shapiro's family studies have defined at least 3 additional genes $\hat{R}^0$, $\hat{\mathfrak{R}}^0$ and $\hat{r}$, with corresponding agglutinogens $\hat{\text{Rh}}_0$, $\hat{\mathfrak{R}}\text{h}_0$ and $\hat{\text{rh}}$.

In 1952 Wiener, Gordon and Cohen[2] reported the results of their early studies on the blood factor **hr″**, and its relationship to the Rh–Hr system. In a series of medicolegal cases of disputed paternity involving Negroes, they encountered 2 instances in which mothers of type Rh$_0$ had babies of type Rh$_2$Rh$_2$. On the assumption that blood factors **rh″** and **hr″** are reciprocally related to each other like factors **M** and **N**, an **rh″**-negative parent could not have an **hr″**-negative child. Thus, these women seemed

*The letter S was chosen by Shapiro for the symbol, because the first 2 reagents of specificity anti-**hr^S** were obtained from 2 patients named Shabalala and Santiago.

to be excluded as being the mothers of their own babies. Therefore, in 1952, there was evidently some defect in the knowledge and understanding of blood factor **hr″**, and Wiener *et al.* decided that it was not safe to include anti-**hr″** reagents in routine Rh–Hr blood tests in medicolegal cases of disputed parentage. In retrospect, it would appear that the explanation for the observations made in 1952 is that the reagent used at that time (and which, unfortunately, is no longer available) must have been of specificity anti-$\mathbf{hr^S}$ instead of anti-**hr″**. With the aid of the reagents now available, the children would almost surely be typed not as Rh_2Rh_2, but as type $Rh_2\hat{r}h$. In support of this view may be cited the observations described by Shapiro, showing that certain commercial reagents labelled anti-**hr″** are really anti-$\mathbf{hr^S}$, while other antisera apparently of specificity anti-**hr″** yield reagents of specificity anti-$\mathbf{hr^S}$ if suitably absorbed, showing that these antisera are actually of specificity anti-**hr″**+$\mathbf{hr^S}$.

Shapiro's new findings extend the series of blood factors of the Rh–Hr system, so that a total of at least 18 such factors are now known (Table 1). Thus, the simple scheme of 6 blood factors C, D, E, c, d, e misrepresents the facts not only by including little d ($\mathbf{Hr_0}$) which has not been proved to exist, but also by omitting 13 blood factors which have actually been found. The scheme is also unrealistic in assuming a simple 1:1 correspondence between agglutinogen and antibody. On the contrary, Shapiro's paper demonstrates again how each Rh–Hr agglutinogen is characterized by multiple serologic factors which are inherited together as a unit by means of corresponding Rh–Hr allelic genes. For example, the gene $\hat{R}^0$ determines

Table 1: List of Known Rh–Hr Antibodies

Antibodies	Blood Factors	Antibodies	Blood Factors
1. Anti-rhesus / Anti-$\mathbf{Rh_0}$	$\mathbf{Rh_0}$ and $\mathfrak{R}\mathbf{h_0}$	10. Anti-$\mathbf{rh^{W_2}}$	$\mathbf{rh^{W_2}}$
2. Anti-**rh′**	**rh′**	11. Anti-$\mathbf{rh^G}$	$\mathbf{rh^G}$
3. Anti-**hr′**	**hr′**	12. Anti-$\mathbf{rh_i}$	$\mathbf{rh_i}$
4. Anti-$\mathbf{rh''}$	$\mathbf{rh''}$	13. Anti-$\mathbf{Rh^A}$	$\mathbf{Rh^A}$
5. Anti-$\mathbf{hr''}$	$\mathbf{hr''}$	14. Anti-$\mathbf{Rh^B}$	$\mathbf{Rh^B}$
6. Anti-$\mathbf{rh^{W_1}}$	$\mathbf{rh^{W_1}}$	15. Anti-$\mathbf{Rh^C}$	$\mathbf{Rh^C}$
7. Anti-**hr**	**hr**	16. Anti-$\mathbf{Rh^D}$	$\mathbf{Rh^D}$
8. Anti-$\mathbf{hr^X}$	$\mathbf{rh^X}$	17. Anti-**Hr**	**Hr**
9. Anti-$\mathbf{hr^V}$	$\mathbf{hr^V}$	18. Anti-$\mathbf{hr^S}$	$\mathbf{hr^S}$

The blood factors have been arranged chronologically in the order of their discovery.

The numbers are not intended as a basis for a new nomenclature, but were suggested by Rosenfield and Kochwa[8] to be used for coding purposes.

a corresponding agglutinogen $\hat{R}h_0$ having the blood factors **Rh_0**, **hr′**, **hr″**, **hr**, **rh^G**, **Rh^A**, **Rh^B**, **Rh^C**, **Rh^D** and **Hr**, but lacking the blood factors **rh′**, **rh″**, **rh^{W1}**, **rh^X**, **hr^V**, **rh^{W2}**, **rh_i**, and **hr^S** (listed in Table 1). Yet, because of its attractive simplicity, the scheme of 6 blood factors and "triple inheritance" continues to be taught. In the past, faith in the C–D–E scheme was so strong that it took the place of scientific knowledge and objective scientific work, causing impressionable investigators to report the discovery of non-existent blood factors like d. Moreover, futile attempts to reconcile the observed facts with the 6 factor scheme have led to fallacies and contradictions.[3, 4] Shapiro has demonstrated in his paper how failure to take into account the blood factor **hr^S** could lead to false exclusions of paternity and maternity in medicolegal cases.

The Committee on Medicolegal Problems of the American Medical Association, in a series of reports[5, 6] has recommended that in medicolegal reports the Rh–Hr nomenclature be used exclusively and that the C–D–E symbols be avoided. Obviously, the true expert must have a thorough knowledge and understanding of the complexities of the Rh–Hr blood types if he is to avoid mistakes, such as falsely excluding parentage through lack of knowledge of the factor **hr^S** described by Shapiro. The use of the C–D–E notations is conducive to such errors, but the true expert will avoid them since he prefers accuracy to simplicity. This vindicates Wiener's assertion[7] that the correct use of Rh–Hr nomenclature may be employed as one of the criteria by which to judge the qualifications of a medicolegal expert.

References

1. Shapiro, M. (1960): *Serology and Genetics of a "New" Blood Factor:* **hr^S**, J. Forensic Med., **7**, 96.
2. Wiener, A. S., Gordon, E. B. and Cohen, L. (1952): *A New Rare Rhesus Agglutinogen*, Amer. J. Human Genet., **41**, 363.
3. Wiener, A. S. (1955): *The Rh–Hr Blood Types: A Budget of Paradoxes*, J. Forensic Med., **3**, 224.
4. Wiener, A. S. (1960): *Another Blood Group Fallacy*, Bull. Amer. Assoc. Blood Banks, **13**, 197.
5. Wiener, A. S., Owen, R. D., Stormont, C. and Wexler, I. B. (1957): *Medicolegal Application of Blood Grouping Tests. A Report of the Committee on Medicolegal Problems of the American Medical Association*, J. Amer. Med. Assoc., **164**, 2036.
6. Wiener, A. S., Unger, L. J. and Sacks, M. S. (1960): *Rh–Hr Blood Types: Present Status, A Special Report of the Committee on Medicolegal Problems*, J. Amer. Med. Assoc., **172**, 1158.
7. Wiener, A. S. (1956): *Blood Grouping Tests in Disputed Parentage: Qualifications of Experts*, J. Forensic Med., **3**, 139.
8. Rosenfield, R. E. and Kochwa, S. (1960): Personal communication.

A. S. Wiener, M.D.
Serological Laboratory,
Office of the Chief Medical Examiner,
New York City

Serology and Genetics of a New Blood Factor: $\mathbf{hr^S}$

Maurice Shapiro, M.B., B.Ch.

The South African Blood Transfusion Service, Johannesburg, South Africa

Investigation of an unusual case of haemolytic disease of the newborn has disclosed further complexities of the Rh–Hr blood group system.

In August 1957, a Bantu woman (Mrs. Shabalala) terminated her ninth pregnancy with the birth of a live son. Four of her previous infants were stillborn and two had died in the first two years of life. Within a few hours of birth, the newborn infant became jaundiced. His blood was typed as Group A rh and the direct antiglobulin reaction was strongly positive. The mother was typed as Group O rh. Her cells gave a negative direct antiglobulin reaction and her serum agglutinated all the samples of a comprehensive panel of test cells maintained in this laboratory for the identification of irregular antibodies. The infant's serum gave similar reactions, as did also an eluate made from his washed cells. The rapidly advancing neo-natal jaundice and the maternal obstetric history indicated an urgent need for exchange transfusion. As it seemed unlikely that compatible blood would be found without a prolonged search, it was decided to perform the transfusion with two units of Group O rh blood. Although both bloods were strongly agglutinated by the infant's serum, the exchange was effected without untoward reaction and the jaundice cleared completely within a few days. At the age of $2\frac{3}{4}$ years the child is physically and mentally normal.

On re-testing with a battery of univalent anti-$\mathbf{Rh_0}$ antisera using papain-treated cells, the bloods of both infant and mother have been shown to be a very low grade type $\mathfrak{Rh}_0$.

The detailed blood group phenotypes of mother, father and child are as follows:

Mrs. Sh: O, $\mathfrak{Rh}_0^v$, MN.Ss, He−, U+, Tj^a+, P+, Le(a+b−), Lu(a−b+), kk, Kp(a−b+), Fy(a−b+), Jk(a+b+), Di^a−, Mi^a−, Vel+, Ca−, I+, Yt^a+, Be^a−, Rm−, Js−.

Mr. Sh: A_1, Rh_2rh^v, MN.ss, He−, U+, P+, Le(a−b+), Lu(a−b+), kk, Kp(a−b+), Fy(a+b+), Jk(a+b+), Di^a−, I+.

Infant (at 3 months): A_1, $\mathfrak{Rh}_0^v$, MN.Ss, He−, U+, P+, Le(a+b+), Lu(a−b+), kk, Kp(a−b+), Fy(a−b+), Jk(a+b+), Di^a−, I+.

Mrs. Shabalala's serum was tested with 1,858 White and 1,749 Bantu bloods, without finding any which were entirely compatible. Tests with ficin-treated cells or by the indirect antiglobulin test were positive in every case, but in 56 Whites (3%) and 106 Bantu (6%) no agglutination occurred with untreated cells suspended in saline. The difference is statistically significant ($x^2=19{\cdot}5$ for 1 d.f.; $p < {\cdot}001$).

The results of random sampling and of the phenotyping tests seemed to eliminate all the known high-frequency antibodies (e.g. anti-**H**, -**Tj^a**, -**k**, -**Yt^a**, -**Vel**, -**I** and -**hr″**). The enhancement of the reactions with enzymes, optimal agglutination at 37° C. and the clinical evidence of erythroblastosis foetalis favoured some form of Rh–Hr sensitization—perhaps a new high-frequency antibody, perhaps a mixture of antibodies. Four commercial anti-**hr″** sera were available to the author at the time; Shabalala's cells reacted strongly with all of them.

Samples of Mrs. Shabalala's blood were sent to several eminent investigators whose advice, encouragement and assistance the author wishes to acknowledge with gratitude.

Drs. A. S. Wiener and L. J. Unger reported that Shabalala's blood reacted negatively with their newly-discovered anti-**Rh^A**, -**Rh^B** and -**Rh^C** sera. They have since found that her cells react negatively also with anti-**Rh^D**. The author prepared an eluate of Shabalala's cells after sensitization with a potent blocking anti-**Rh$_0$** serum. This yielded a specific anti-**Rh$_0$** reagent. Her Rh phenotype can therefore be designated $\mathfrak{R}h_0^{abcd}$.

Drs. R. R. Race and Ruth Sanger reported that Shabalala's serum did not agglutinate the thawed blood samples of two related $\overline{\overline{R}}h_0$(–D–/–D–) North American individuals; they also found that Shabalala's cells were not agglutinated by the serum of an American negress (Davis) which had been sent to them by Dr. Eloise Giblett and which appeared to contain a similar high-frequency antibody.

Dr. Dorothy Parkin subjected Shabalala's serum to various absorptions and obtained results which suggested that it contained a mixture of Rh–Hr antibodies.

Fresh samples of Shabalala's and Davis' bloods were exchanged with Dr. Giblett. The serum of each patient was found to react weakly with the papain-treated cells of the other, but both bloods were compatible in saline, albumin and by the indirect Coombs test. Dr. Giblett confirmed that $\overline{R}h_0$ cells were not agglutinated by the sera of either patient. She also mentioned that Davis' cells were not agglutinated by certain of her anti-**hr″** sera.

Samples of Shabalala's blood were also exchanged with Dr. R. E. Rosenfield who had recently found an unidentified high-frequency antibody in the blood of a Puerto Rican woman (Santiago) who, similarly, had been sensitized by pregnancy. The bloods of Shabalala and Santiago were found to be virtually identical in their Rh phenotypes, and entirely compatible with each other. Rosenfield observed that both bloods gave negative reactions with certain of his anti-**hr″** sera.

At this stage it was clear that the antibody or antibodies in the Shabalala serum were of Rh–Hr specificity with the focus of attention on the unusual **hr″** reactions.

Methods

The detailed investigation of Shabalala's blood involved numerous absorptions, elutions and titrations with cells of various Rh–Hr specificities. The initial absorptions in each case were performed with a double volume of untreated washed cells. For all subsequent absorptions, the packed cells were treated for 20 minutes with a half volume of 0·5% bromelin (Wallerstein Laboratories, New York) and then washed twice. Errors in titration from dilution in repetitive absorptions were minimized by the use of a high speed centrifuge (20,000 r.p.m.) at every stage in the preparation of absorbed sera and eluates. Eluates were made by Dr. W. Weiner's method[1] with the following modifications: The sensitized washed cell ghosts were resuspended in distilled water, spun-frozen and lyophilized. The antibody in these dried cells was found to be stable for many months. The final eluate was prepared in each case by mixing 1 gm. of the powder with 5 ml. of normal saline, heating the suspension in plastic cups at 60° C. for 15 minutes and separating the supernatant rapidly after spinning at high speed in a heated centrifuge head.

Notations

The investigation has disclosed that Shabalala's serum contains two antibodies, here designated as anti-**Hr** and anti-**hr**^{S}.

Anti-**Hr** is regarded by the author as lacking the necessary specificity to define new alleles with precision. Specific notations to indicate the presence or absence of **Hr** in the phenotype are therefore not considered necessary at this stage. The anti-**Hr** component of Shabalala's serum appears to be similar to the antibody (or spectrum of related antibodies) formed by type $\overline{\text{R}}\text{h}_0$ individuals.

The second antibody, anti-**hr**^{S} reacts like anti-**hr**″ except that certain bloods which are **hr**″ positive give negative reactions with anti-**hr**^{S}. Anti-**hr**^{S} is contained in most, if not in all, sera hitherto regarded as of anti-**hr**″ specificity. Bloods reacting with anti-**hr**″ but not with anti-**hr**^{S} will here be designated with a caret, e.g. $\hat{\text{R}}\text{h}_0$ for the phenotype, $\hat{R}^0$ for the gene. Wiener originally used the designation Rh_0^u and later $\overline{\text{R}}\text{h}_0$ for bloods lacking the pair **rh**″ and **hr**″. As will be shown, bloods lacking the factor **hr**^{S} occur fairly frequently in South African Bantu, but no example of an Rh_0 blood lacking **hr**″ has yet been encountered. This suggests that human bloods previously designated $\overline{\text{R}}\text{h}_0$ were really $\hat{\text{R}}\text{h}_0$. Now that the factors **hr**″ and **hr**^{S} can be clearly distinguished with antibodies specific for each, the existence of type $\overline{\text{R}}\text{h}_0$ bloods lacking all trace of **hr**″ as well as **hr**^{S} needs to be confirmed. If such bloods exist, they must be very rare indeed.

Absorption and Elution Experiments

Table I shows the results of absorptions and elutions of a 'standard' anti-**hr**″ serum (Dade) and of Shabalala's serum. These experiments disclose that the 'standard' anti-**hr**″ serum employed can be fractionated into two specific antibodies—anti-**hr**^{S} and anti-**hr**″. Anti-**hr**^{S} remains in

Table I: Absorption and Elution Studies

Test Cells		I *Anti-*hr^S *(Commercial, Dadé)*	II I *Absorbed with Shabalala Cells*	III *Eluate from* II	IV *Shabalala Serum*	V *Shabalala Serum Absorbed with* Rh_2Rh_2 *Cells*	VI *Eluate from* V	VII *Eluate* VI *Absorbed with* rh *Cells*	VIII *Shabalala Serum Absorbed with* rh *Cells*	IX *Eluate from* VIII	X *Eluate* IX *Absorbed with* Rh_2Rh_2 *Cells*	XI* *Shabalala Serum Absorbed with* Rh_1Rh_1 *Cells*	XII *Eluate from* XI	XIII *Eluate* XII *Absorbed with* Rh_2Rh_2 *Cells*
Shabalala	$\hat{R}h_0$	51 *75*	0 *0*	11 *34*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*
Lepota	$\hat{R}h_0$	18 *59*	0 *0*	8 *18*	5 *13*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*	0 *0*
Mabeca	$Rh_2\hat{r}h$	21 *60*	0 *0*	5 *33*	62 *66*	0 *0*	18 *33*	0 *0*	0 *0*	1 *14*	0 *0*	0 *1*	0 *5*	0 *0*
	Rh_2Rh_2	0 *0*	0 *0*	0 *0*	38 *56*	0 *0*	22 *49*	0 *0*	0 *0*	0 *14*	0 *0*	0 *0*	0 *5*	0 *0*
	Rh_1Rh_2	30 *67*	4 *37*	6 *20*	44 *57*	14 *37*	19 *44*	0 *0*	0 *0*	13 *32*	8 *21*	0 *5*	0 *19*	1 *1*
	Rh_1Rh_1	47 *76*	24 *37*	13 *36*	31 *68*	6 *36*	0 *21*	0 *0*	0 *0*	9 *31*	8 *17*	0 *0*	9 *26*	1 *3*
	rh	39 *68*	26 *61*	8 *19*	44 *73*	26 *61*	5 *30*	0 *0*	0 *0*	32 *46*	26 *42*	0 *17*	23 *31*	21 *25*
	Rh_0	31 *60*	21 *47*	7 *24*	30 *58*	20 *46*	3 *21*	0 *0*	0 *0*	22 *41*	21 *36*	0 *5*	18 *26*	9 *11*

Note: The absorptions in each case were performed as follows: First absorption with double volume of untreated washed cells; three further absorptions with double volumes of bromelin-treated cells. Eluates were prepared from the cells used for the first absorption.

For scoring of the agglutination reactions in two-fold dilutions, the following formula was adopted: 4+=10; 3+=8; 2+=5; 1+=3; ±=1.

In each column the left hand figures are for untreated cells in saline; right hand figures (*italicized*) are for bromelin-treated cells.

*Two further absorptions with bromelin-treated cells were required to remove all traces of antibody.

the serum after absorption with Shabalala's cells (Column II). (The same result was obtained after absorption with the cells of Lepota ($\hat{R}h_0$) or Mabeca ($Rh_2\hat{r}h$)). The other antibody, anti-**hr″** is recovered in the eluate of Shabalala's cells sensitized with Dade anti-**hr″** serum (Column III). (The same result was obtained when $\hat{R}h_0$ or $Rh_2\hat{r}h$ cells were used for preparation of the eluate.) Several examples of commercial anti-**hr″** sera have since been examined. All have been found to contain anti-$\mathbf{hr}^S$ but the amount of pure anti-**hr″** recoverable as an eluate or detectable by agglutination of $\hat{R}h_0$ cells has varied: a few contain potent anti-**hr″**; some contain very little anti-**hr″**; others contain no detectable anti-**hr″** at all.

After absorption of Shabalala's serum with Rh_2Rh_2 cells (Column V) an antibody is obtained which reacts in all respects like the anti-$\mathbf{hr}^S$ reagent obtained by absorption of anti-**hr″** serum with $\hat{R}h_0$ or $Rh_2\hat{r}h$ cells. The antibody contained in the eluate of the Rh_2Rh_2 cells used for the absorption of the Shabalala serum is regarded as anti-**Hr.** This eluate has a marked affinity for cells containing **rh″.** In the initial experiments, this feature was interpreted as indicating that Shabalala's serum contained anti-**rh″** in addition to other antibodies. However, repeated absorptions of this eluate with rh cells removed all traces of antibody (Column VII). Similarly, both the anti-$\mathbf{hr}^S$ and the anti-**Hr** antibodies in Shabalala's native serum could be neutralized completely by absorption with rh cells (Column VIII). It was found that absorption of Shabalala's serum with rh cells removed anti-$\mathbf{hr}^S$ more readily than anti-**Hr** and the incompletely absorbed serum reacted more strongly with cells containing **rh″.** This result was similarly misinterpreted to indicate the presence of anti-**rh″** in the serum. Just as the eluate from Rh_2Rh_2 cells shows an affinity for cells containing the **rh″** factor, so eluates of rh or Rh_1Rh_1 cells sensitized with Shabalala's serum show an affinity for cells lacking **rh″** in one or both agglutinogens (i.e. rh, Rh_0, Rh_1Rh_1 and Rh_1Rh_2).

The eluates of rh and Rh_1Rh_1 cells sensitized with Shabalala serum contain both anti-$\mathbf{hr}^S$ and anti-**Hr.** When these eluates were absorbed with Rh_2Rh_2 cells, anti-$\mathbf{hr}^S$ was left (Columns X and XIII). Absorption of an eluate of Rh_1Rh_1 cells with Rh_2Rh_2 cells (Column XIII) yielded a reagent which appeared to react preferentially with rh and Rh_0 cells, simulating anti-**hr.**

Not all $\hat{R}h_0$ cells are compatible with Shabalala serum. Lepota cells, for example, were weakly agglutinated, as were two other Bantu $\hat{R}h_0$ samples found during the course of this investigation. However, the eluates of Rh_2Rh_2, Rh_1Rh_1 and rh cells sensitized with Shabalala serum failed to agglutinate $\hat{R}h_0$ bloods. The significance of this finding is not understood. It may be that such bloods have too few **Hr** combining sites to be visibly affected by the antibody recovered in the eluate, which is, of course, weaker than in the native serum.

The Serological Specificity of Anti-**hr^S**

Anti-**hr^S** and anti-**hr″** have not hitherto been differentiated. As has been shown, anti-**hr^S** can be isolated from certain 'standard' anti-**hr″** sera (e.g. Dade, Biotest) by absorption with $\hat{R}h_0$ or $Rh_2\hat{r}h$ cells. Although Shabalala's own cells are strongly **hr″** positive, she has herself produced a potent anti-**hr^S** antibody.

Since the frequency of $Rh_2\hat{r}h$ is greater than that of Rh_2Rh_2 in South African Bantu, it may well be that the majority of so-called anti-**hr″** antibodies produced by Bantu are of anti-**hr^S** specificity only. This may also be generally true of African negroes and their descendants.

The sensitization of an **hr″** positive individual with the production of an anti-**hr″**-like antibody is reminiscent of the examples of anti-**Rh$_0$**-like antibody produced by **Rh$_0$** positive individuals. The first example of such a paradoxical sensitization was reported by Shapiro[2] in 1951. Other examples have since been reported by Argall,[3] by Rosenfield et al.[4] and by Wiener et al.[5] Unger and Wiener et al.[6, 7] have classified the anti-**Rh$_0$**-like antibodies detected in their own series as anti-**Rh^A**, anti-**Rh^B**, anti-**Rh^C** and anti-**Rh^D**.

By analogy, **hr^S** may be regarded as the first of a series of 'associated' blood factors related to **hr″** in the same way as **Rh^A**, **Rh^B** . . . etc., are related to **Rh$_0$**.

However, there is evidence that the relationship of anti-**hr^S** to anti-**hr″** may be on a different plane. Whereas none of the subsidiary anti-**Rh** antibodies has so far been shown to be the same as one already discovered, the anti-**hr^S** reagents isolated from the Shabalala and Santiago sera are well-defined and indistinguishable in their reactions. Similarly, absorption of any 'standard' anti-**hr″** serum with any $\hat{R}h_0$ or $Rh_2\hat{r}h$ blood yields a reagent which is specifically anti-**hr^S**. The status of the **hr^S** factor may therefore be analogous to that of the other primary Rh–Hr factors (e.g. **rh′**, **hr′**, **Rh$_0$**, etc.) and its co-existence with anti-**hr″** in most so-called anti-**hr″** sera may have no other serological significance than the co-existence of, say, anti-**Rh$_0$** and anti-**rh′** in anti-**Rh$_0'$** sera.

If bloods are discovered which contain **hr^S** but no **hr″**, this will strongly support the latter hypothesis and may require re-designation of the factor (say **hr‴**) and of the phenotypes, genes, etc., in order to indicate the presence, rather than the absence, of the **hr^S** factor. Whichever explanation proves to be correct, there is no difficulty in accommodating the new alleles which are defined by this factor in terms of Wiener's serological concepts and theory of inheritance of the Rh–Hr blood groups.

Population Studies with Anti-**hr^S**

1,390 South African Bantu and 1,779 Whites were examined with an anti-**hr^S** reagent prepared by absorption of Shabalala serum with Rh_2Rh_2 cells. All negative reactors were re-tested with an anti-**hr^S** reagent prepared

from a 'standard' anti-**hr″** serum (Dade) by absorption with Shabalala cells and with the neat 'standard' anti-**hr″** serum. The results are shown in Table II.

Table II: Distribution of Bloods Lacking **hr^S** in Whites and Bantu

Phenotype	*Bantu*	*Whites*
$\hat{R}h_0$	1	0
$\hat{\mathfrak{R}}h_0$	1	0
$Rh_2\hat{r}h$	11	3
Rh_2Rh_2	8	36
Others	1,369	1,707
Total	1,390	1,743

A further investigation is now under way using a 'pure' anti-**hr″** reagent prepared as an eluate from Shabalala's cells sensitized with 'standard' anti-**hr″** serum (Dade) in parallel with absorbed Shabalala (anti-**hr^S**) serum. It is possible that this investigation will reveal that certain rare bloods contain **hr^S** but no **hr″**. The results will be reported in due course.

The population and family studies so far carried out by the author have revealed an absence of **hr^S** only in certain rare rh and Rh_0 agglutinogens. $Rh_2\hat{r}h$ includes genotypes $R^2\hat{R}^0$, $R^2\hat{\mathfrak{R}}^0$ and $R^2\hat{r}$; type $\hat{R}h_0$ includes the genotypes $\hat{R}^0\hat{R}^0$, $\hat{R}^0\hat{\mathfrak{R}}^0$, and $\hat{R}^0\hat{r}$; type $\hat{\mathfrak{R}}h^0$ includes $\hat{\mathfrak{R}}^0\hat{\mathfrak{R}}^0$ and $\hat{\mathfrak{R}}^0\hat{r}$; while type $\hat{r}h$ must be $\hat{r}\hat{r}$. There is evidence in the literature[8, 9] to suggest that the **hr^S** factor may also occasionally be lacking in other agglutinogens (e.g. Rh_1, Rh_1^w). In the case of the Rh_1 agglutinogen the anomaly would be manifest as $\hat{R}h_1Rh_2$, $\hat{R}h_1\hat{r}h$ or $\hat{R}h_1\hat{R}h_1$ phenotypes.

On the basis of the observed frequencies of the Rh–Hr types in South African Bantu,[2] the present author has estimated the frequency of the R^2 gene as approximately 6% with a negligible frequency for the r'' gene. Thus 5 R^2R^2 bloods would be expected in the present series of 1,390 Bantu bloods examined (Table II). In fact, 8 type Rh_2Rh_2 bloods were observed, which is within expected limits, assuming all to be genotype R^2R^2. A further 13 bloods were negative with anti-**hr^S** but positive with the standard anti-**hr″** serum. Assuming that the absence of the **hr^S** factor is genetically determined only by the $\hat{R}^0$, $\hat{\mathfrak{R}}^0$ and $\hat{r}$ genes, the combined frequency of these genes is approximately the same as for the R^2 gene itself (ca. 6%). These '**hr^S**-deficient' genes therefore constitute a sizeable proportion of the total Rh genes in the Bantu.

All three $Rh_2\hat{r}h$ individuals in the Caucasian group were found to be of genotype $R^2\hat{R}^0$. Since type Rh_0 in South African Whites has a frequency of under 3%, this exclusive association of the rare '**hr^S**-deficiency' with type Rh_0 in three consecutive examples is highly significant and supports the possibility that the deficiency is not shared proportionately by all agglutinogens containing **hr″**, as might be expected if **hr^S** was a subsidiary blood factor 'associated' with **hr″**.

Shapiro

abalala
shown

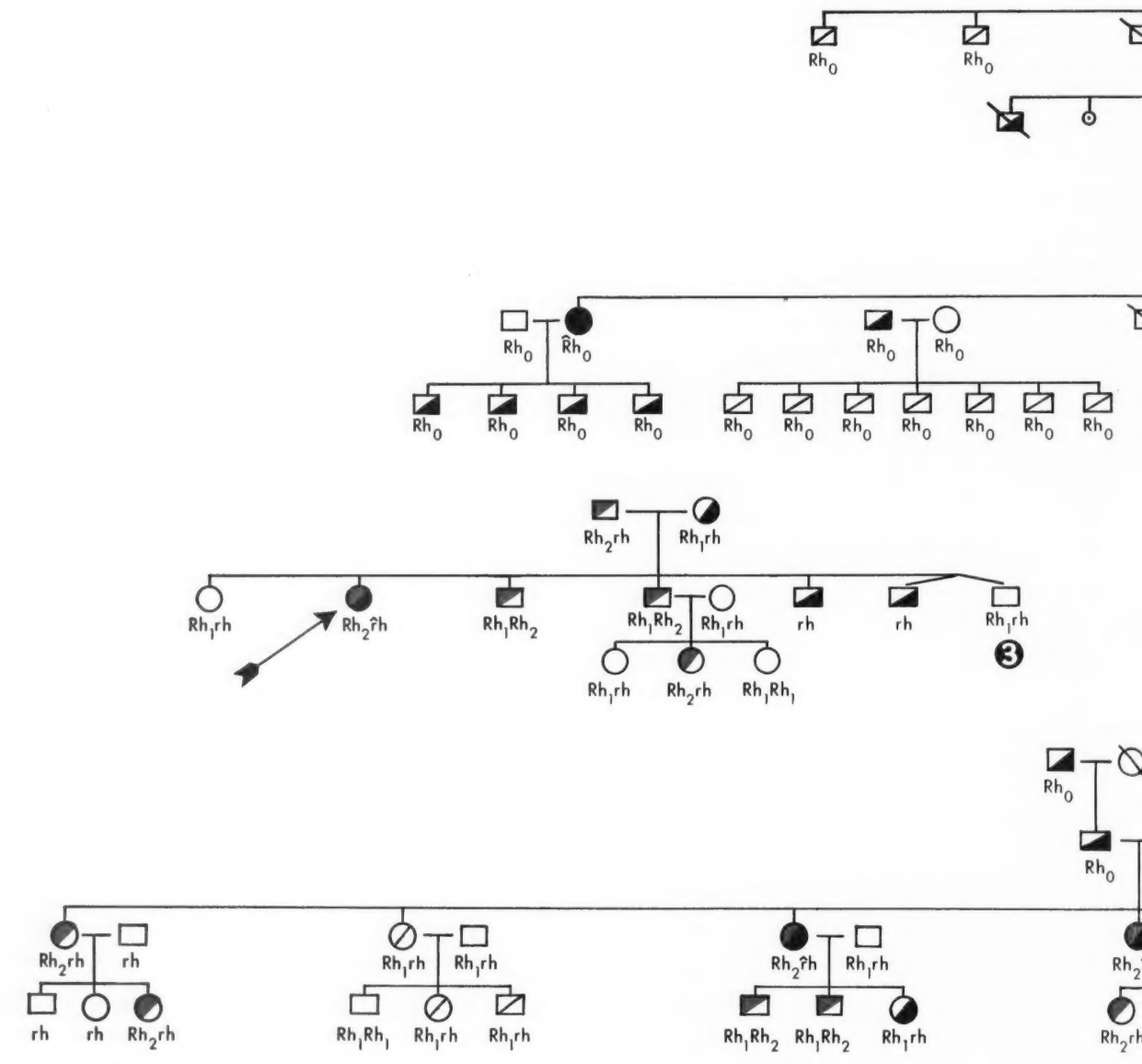
eagent
ndard'
serum.
bloods

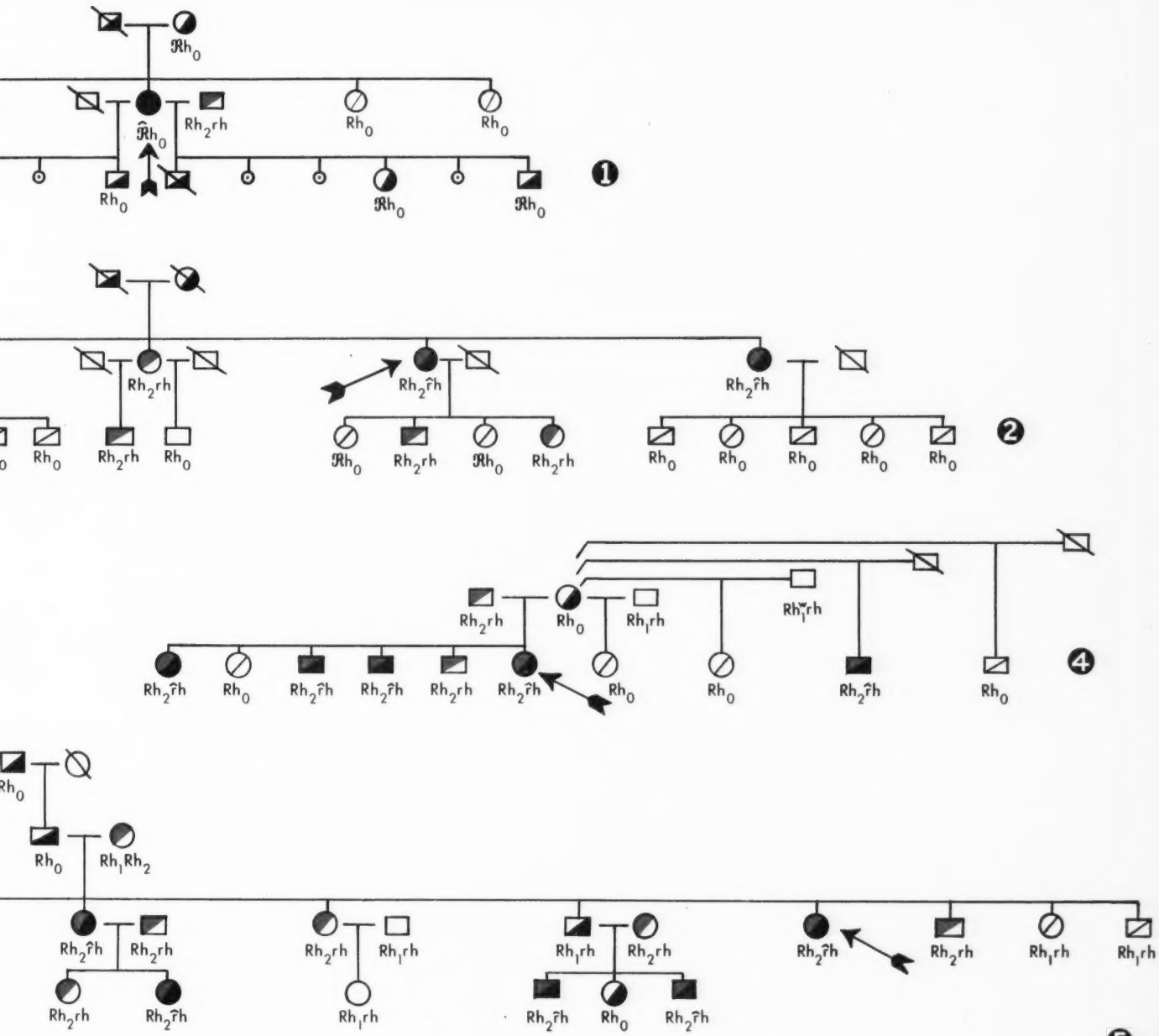
r have
ogens.
es the
while
nat the
g. Rh_1,
anifest

South
the R^2
gene.
1,390
s were
notype
e with
factor
nbined
^{2} gene
izeable

to be
quency
' with
pports
by all
sidiary

Medicine


Rh0
Rh0
Rh0
R̂h0
Rh0
Rh0
Rh0
Rh0
Rh0
Rh0
Rh0
Rh0
Rh0
Rh0
Rh0
Rh2rh
Rh1rh
Rh1rh
Rh2r̂h
Rh1Rh2
Rh1Rh2
Rh1rh
rh
rh
Rh1rh
3
Rh1rh
Rh2rh
Rh1Rh1
Rh0
Rh0
Rh2rh
rh
rh
rh
Rh2rh
Rh1rh
Rh1rh
Rh1Rh1
Rh1rh
Rh1rh
Rh2r̂h
Rh1rh
Rh1Rh2
Rh1Rh2
Rh1rh
Rh2
Rh2rh
Key:
Homozygous, e.g. R̂oR̂o
Carrier of one R̂o (or r̂) gene


Rh0
Rh2rh
Rh0
Rh0
Rh0
Rh0
Rh0
1
Rh2rh
Rh2rh
Rh2rh
Rh0
Rh0
Rh2rh
Rh0
Rh0
Rh2rh
Rh0
Rh0
Rh0
Rh0
Rh0
2
Rh2rh
Rh0
Rh1rh
Rh1rh
Rh2rh
Rh2rh
Rh2rh
Rh2rh
Rh2rh
Rh0
Rh0
Rh2rh
Rh0
4
Rh0
Rh0
Rh1Rh2
Rh2rh
Rh2rh
Rh2rh
Rh1rh
Rh1rh
Rh2rh
Rh2rh
Rh2rh
Rh0
Rh2rh
Rh2rh
Rh1rh
Rh1rh
5
Possible carrier of one R0 (or r) gene
Carrier of one R2 gene

Family Studies

An attempt was made to obtain samples of blood from members of the families of all $Rh_2\hat{r}h$ and $\hat{R}h_0$ individuals found in random sampling.

The information regarding the mode of inheritance of the **hr**^{S} blood factor supplied by the Bantu kindreds is unsatisfactory in many respects: great difficulty was experienced in most cases in obtaining blood samples because of dispersal of members of the families; in many instances the question of the paternity was clearly unreliable; and the very high incidence of the R^0 gene in the Bantu makes it impossible in most cases to discriminate by blood tests alone between R^0, $\mathcal{R}^0$ and r in the genotypes.

Mrs. Shabalala's own kindred is shown in Fig. 1. The anomalous gene in this case is undoubtedly very low grade $\hat{\mathcal{R}}^0$.

Fig. 2 shows the likely mode of inheritance in the kindred of the Mabeca family. Her eldest sister is type $\hat{R}h_0$ (probable genotype $\hat{R}^0\hat{R}^0$).

Fig. 3 shows a Bantu family in which the anomaly is apparently being transmitted by an $\hat{r}$ gene.

The kindreds of the three $Rh_2\hat{r}h$ propositi of European descent have provided much more helpful data. In all three of these, one of the parents was type Rh_0 and the **hr**^{S}-deficiency was clearly transmitted by an $\hat{R}^0$ gene.

Fig. 4 shows the progeny of 5 matings of a White woman. Her $\hat{R}^0$ gene has apparently been transmitted to all but one of her 10 children and the **hr**^{S} deficiency is discernible in five whose genotypes are $R^2\hat{R}^0$.

One magnificent kindred, consisting of over 100 White persons in 4 generations, provided most information. For the sake of brevity, only that portion of the pedigree has been presented (Fig. 5) which provides objective evidence of the mode of transmission of the anomalous $\hat{R}^0$ gene.

The results of random sampling and of family studies confirm that the Rh_2 agglutinogen contains no **hr**^{S} blood factor. The **hr**^{S} deficiency in the other genes (e.g. $\hat{R}^0$, $\hat{r}$) is manifested only when the anomalous genes are paired as in the combinations $\hat{R}^0\hat{R}^0$, $\hat{R}^0\hat{r}$, etc., except when one anomalous gene is combined with an R^2 gene, as in the genotypes $R^2\hat{R}^0$ or $R^2\hat{r}$.

Forensic Significance of **hr**^{S}

Many paradoxical situations hitherto attributed to illegitimacy or to 'chromosome deletions' will doubtless be resolved by the recognition of the distinction between **hr**^{S} and **hr**″. The factor **hr**″ can be defined as the genetic reciprocal of **rh**″. Except perhaps in such exceptional instances as type $\bar{\bar{R}}h_0$ bloods, **hr**″ is invariably present when **rh**″ is absent. This reciprocal relationship with **rh**″ does not apply as strictly in the case of **hr**^{S}, particularly in Negroes. As has been stated, all so-called anti-**hr**″ sera so far tested contain anti-**hr**^{S} but some contain little if any anti-**hr**″. Fallacious conclusions may therefore be drawn if an anti-**hr**^{S} serum is employed as an

anti-**hr**″ reagent. In the case of the South African Bantu, the diagnosis of homozygosity in respect of the **hr**″ factor will be wrong about two times in three if an anti-**hr**^{S} serum is used. Table III lists some examples of fallacious exclusions of parentage and their possible explanations.

Table III: Examples of Fallacious Exclusions of Parentage when Using Anti-**hr**^{S} as an Anti-**hr**″ Serum

	Parents	*Child*	*Possible Explanation*
1.	Rh_0 × Rh_2Rh_2	→ Rh_2Rh_2	$\hat{R}^0r$ × R^2R^2 → $R^2\hat{R}^0$
2.	Rh_0 × Rh_2rh	→ Rh_2Rh_2	$\hat{R}^0r$ × R^2r → $R^2\hat{R}^0$
3.	Rh_0 × Rh_1Rh_2	→ Rh_2Rh_2	$\hat{R}^0r$ × R^1R^2 → $R^2\hat{R}^0$
4.	rh × Rh_2Rh_2	→ rh	rr × $R^2\hat{r}$ → $r\hat{r}$
5.	rh × Rh_2Rh_2	→ Rh_0	rr × $R^2\hat{R}^0$ → $\hat{R}^0r$
6.	rh × Rh_2rh	→ Rh_2Rh_2	$r\hat{r}$ × R^2r → $R^2\hat{r}$
7.	rh × Rh_1Rh_2	→ Rh_2Rh_2	$r\hat{r}$ × R^1R^2 → $R^2\hat{r}$
8.	rh′rh × Rh_2Rh_2	→ Rh_0, Rh_1rh	$r'r$ × $R^2\hat{R}^0$ → $\hat{R}^0r$, $r'\hat{R}^0$
9.	rh′rh × Rh_1Rh_2	→ Rh_2Rh_2	$r'\hat{r}$ × R^1R^2 → $R^2\hat{r}$
10.	rh″rh × Rh_2Rh_2	→ Rh_0	$r''r$ × $R^2\hat{R}^0$ → $\hat{R}^0r$
11.	Rh_1Rh_2 × Rh_2Rh_2	→ Rh_1rh	R^1R^2 × $R^2\hat{R}$ → $R^1\hat{R}^0$
12.	Rh_2Rh_2 × Rh_1Rh_2	→ Rh_1Rh_1	$\hat{R}^1R^2$ × R^1R^2 → $\hat{R}^1R^1$

In describing Rh_o^u as 'a new rare Rhesus agglutinogen' in 1952, Wiener[10] cautioned against the use of anti-**hr**″ sera in tests for the exclusion of parentage. He cited two examples of atypical Rh–Hr reactions in mother and child; in both, the mother was type Rh_0 and the child type Rh_2Rh_2 (cf. Examples 2 and 3 of Table III). He also described a type Rh_0 blood which reacted negatively with anti-**hr**″ serum. It seems reasonably certain that the anomalous results reported by Wiener were due to the fact that the serum which he was using contained only anti-**hr**^{S} and no anti-**hr**″.

Now that the factors **hr**″ and **hr**^{S} can be separately identified with reagents specific for each, the determination of the presence or absence of the **hr**″ factor assumes a new significance in the investigation of cases of disputed parentage and a more confident interpretation of anti-**hr**″ reactions becomes possible. However, it needs to be emphasized that in order to qualify as a 'standard' anti-**hr**″ reagent, a serum should be shown to have a minimum prescribed titre with $\hat{R}h_0$ and $Rh_2\hat{r}h$ bloods.

Summary

A new antibody, anti-**hr**^{S} has been found in the serum of a Bantu woman (Shabalala).

The antibody is contained in most, if not all, so-called anti-**hr**″ sera, either alone or together with true anti-**hr**″.

The serology, distribution in Caucasians and Bantu and the genetics of the **hr**^{S} blood factor have been studied.

Recognition of this blood factor explain certains anomalous findings which have previously been attributed to illegitimacy or to 'chromosome deletions'.

In addition to those already mentioned, the author wishes to express his gratitude to numerous colleagues who assisted with the procurement of blood specimens for family studies.

The author is particularly indebted to Miss Marjorie E. le Roux and to Miss Alice C. Irvine for their diligent assistance with the serological tests.

References

1. Weiner, W.: *Eluting rh-Cell Antibodies: A Method and its Application.* Brit. J. Haemat., 1957, **3**, 276.
2. Shapiro, M.: *The* ABO, M–N, P *and* Rh *Blood Group Systems in the South African Bantu.* S. Afr. Med. J., 1951, **25**, 187.
3. Argall, C. I., Ball, J. M. and Trentleman, E.: *Presence of* Anti-D *Antibody in the Serum of a* D *Patient.* J. Lab. Clin. Med., 1953, **41**, 895.
4. Rosenfield, R. E., Haber, G. and Gibbel, N.: *A New* Rh *Variant.* Proc. Sixth Congr. Internat. Soc. Blood Transfusion, 1958, p. 90.
5. Wiener, A. S., Geiger, J. and Gordon, E. B.: *Mosaic Structure of the* **Rh$_0$** *Factor.* Exp. Med. Surg., 1957, **15**, 75.
6. Unger, L. J., Wiener, A. S. and Katz, L.: *Studies on the Blood Factors* **Rh^A**, **Rh^B**, **Rh^C**. J. Exp. Med., 1959, **110**, 495.
7. Sacks, M. S., Wiener, A. S., Jahn, E. F., Spurling, C. L. and Unger, L. J.: *Iso-sensitization to a New Blood Factor* **Rh^D**, *with Special Reference to its Clinical Importance.* Ann. Int. Med., 1959, **51**, 740.
8. Gunson, H. H. and Donohue, W. L.: *C^{W}D-/C^{W}D- in a Canadian Family.* Vox Sang., 1957, **2**, 320.
9. Sussman, L. N.: *Pitfalls of Paternity Blood Grouping Tests.* Amer. J. Clin. Path., 1960, **33**, 406.
10. Wiener, A. S., Gordon, E. B. and Cohn, L. *A New Rare Rhesus Agglutinogen.* Amer. J. Human Genet., 1952, **4**, 363.

Simple Determination of the Amount of Alcohol Ingested by an Individual

N. Sapeika, B.A., M.D., Ph.D.

Department of Physiology and Pharmacology, University of Cape Town

A Table is provided which permits easy and rapid determination of:

(*a*) The minimal quantity of alcoholic drinks an individual must have taken.

This is calculated from the value for the total amount of alcohol absorbed in the body, which is determined from the body weight and the blood alcohol concentration (see *Calculation* later);

(*b*) The approximate blood alcohol concentration that may result after alcohol has been taken;

(*c*) The approximate time required for the removal of alcohol from the body.

General Considerations

The alcohol content of certain beverages, expressed in millilitres (ml.), is approximately as follows:

Brandy, Whisky, Gin: Strength 43%, by volume; 1 tot, i.e. 35 ml., contains 15 ml. alcohol.

Dry Wines (*Natural; Unfortified*): Strength 11–13%, by volume; 1 bottle, i.e. 26⅔ fluid ounces (12%) contains 90 ml. alcohol (approximately). In this group are local brands such as Golden Blossom, Golden Castle, Golden Jubilee, Mardi Gras, Oom Tas, Virginia; also the Rhine wines, sparkling wines, claret and rosé types, and boer wine (vaaljapie).

Sweet Wines (*Fortified*): Strength 16.6–20.1%, by volume; 1 bottle, i.e. 26⅔ fluid ounces (18%) contains 135 ml. alcohol (approximately). In this group are Sherry, Muscadel, Port, Jerepigo, Worcester Hock. Madeira, Malaga and Marsala are other fortified wines.

For certain purposes, in South Africa and Britain, the alcohol content is indicated in degrees proof spirit: 7 *degrees proof spirit equals* 4% *alcohol by volume.* Thus a South African wine of 21 degrees contains the same amount of alcohol as a Continental wine of 12% strength.

Beers, Ales: Strength 4.0–4.4%, by volume; 1 'pint,' i.e. 13⅓ fluid ounces (4%) contains 15 ml. alcohol (approximately); 1 'quart,' i.e. 26⅔ fluid ounces (4%) contains 30 ml. alcohol (approximately).

Stout: Strength 5%, by volume; 1 'pint,' i.e. 13⅓ fluid ounces (5%) contains 19 ml. alcohol (approximately).

Liqueurs: The alcohol content ranges from a minimum of 30%, e.g. peppermint, aniseed, ginger, van der Hum contain approximately 31.5%; Kümmel approximately 45.5%.

Calculation: The Method of Estimation of the Total Minimal Amount of Alcohol (Absorbed) in the Body. This is shown in the following example.

A man 160 lb. in weight with 0.1% alcohol in the blood must have ingested (absorbed) at least 60 ml. alcohol. The weight of the body fluids and the soft tissues is taken as two thirds of the body weight.

Thus: $160 \times \frac{2}{3} \times 0{\cdot}1\%$
$= 0{\cdot}1066$ lb. alcohol absorbed in the body
$= 1{\cdot}705$ oz. by weight
$= 2{\cdot}132$ fluid ounces by volume (specific gravity of alcohol 0·8)
$= 60{\cdot}5$ ml. alcohol (1 fluid ounce = 28·4 ml.)

The values in the Table have been calculated on this basis.

Methods of Estimation

(*a*) *To Estimate the Minimum Amount of Alcoholic Beverage (Drinks) Ingested*:

Rule: 'The value (in the Table) for the total amount of alcohol estimated in the body is divided by the factor representing the amount of alcohol in a particular alcoholic beverage.'

Amount of Drink Ingested	*The Total Amount of Alcohol in the Body, Divided by:*
Tots of Brandy, Whisky, or Gin	15
Bottles of Sweet Wine (18%)	135
Bottles of Dry Wine (12%)	90
Pints of Beer (4%)	15
Pints of Stout (5%)	19

Note: Beers, ales, and stout are supplied as a 'reputed pint' (13⅓ fluid ounces) or 'reputed quart' (26⅔ fluid ounces), and wine by the 'bottle' (26⅔ fluid ounces).

Example: In a man weighing 160 lb., who has a blood alcohol concentration of 0.1%, the total amount of alcohol absorbed in the body is 60 ml. (see *Calculation* above). This amount represents the absorption of alcohol from 4 tots of brandy, whisky or gin, two thirds of a bottle of dry wine (12%), four ninths of a bottle of sweet wine (18%), or 4 pints of beer.

(*b*) *To Estimate the Blood Alcohol Concentration Likely to Result from the Ingestion of a Known Number of Drinks*:

Rule: 'The total amount of alcohol in the drinks, considered in relation to the body weight, gives the blood alcohol concentration (as shown in the Table), provided all the alcohol is absorbed.'

Example: In a man weighing 160 lb. the total amount of alcohol in 4 tots of brandy, whisky or gin, two thirds of a bottle of dry wine (12%), four ninths of a bottle of sweet wine (18%) or 4 pints of beer (4%) is 60 ml. and the blood alcohol concentration may reach 0.1%, provided all the alcohol is absorbed.

(*c*) *To Estimate the Approximate Time in Hours Required for Complete Removal of Alcohol from the Body*:

Rule: 'The value for the total amount of alcohol absorbed in the body is divided by 10.' This is due to the fact that alcohol is oxidized slowly at the rate of about 10 ml. per hour.

Example: If the total amount of alcohol absorbed in the body is 60 ml. it will take 6 hours for its complete removal.

Summary

A Table is provided from which certain easy estimations can be made.

(*a*) The total minimal amount of alcohol absorbed from a certain number of drinks can be directly read from the Table, if the blood alcohol concentration and the body weight of an individual are known. Dividing this total amount by certain factors gives the equivalent amount of alcoholic beverage the individual must have ingested.

(*b*) The blood alcohol concentration that may result after drinking alcohol can be read from the Table, if the amount of drink ingested and the body weight are known.

(*c*) The approximate time that must elapse for complete removal of alcohol from the body can easily be determined.

Table
Total Amount of Alcohol in the Body (in Millilitres)

Weight of Subject (in lb.)	Per cent Alcohol in the Blood (Grammes per 100 ml.)									
	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·1
100	3·7	7·5	11·2	15·0	18·9	22·5	26·2	30·0	33·7	37·8
110	4·2	8·3	12·5	16·6	20·8	25·0	29·1	33·3	37·4	41·6
120	4·5	9·1	13·6	18·2	22·7	27·2	31·8	36·3	40·9	45·4
130	4·9	9·8	14·8	19·7	24·6	29·5	34·4	39·4	44·3	49·2
140	5·3	10·6	15·9	21·2	26·5	31·8	37·1	42·4	47·7	53·0
150	5·7	11·4	17·0	22·7	28·4	34·1	39·8	45·4	51·1	56·8
160	6·0	12·1	18·0	24·2	30·3	36·3	42·3	48·4	54·5	60·5
170	6·4	12·8	19·3	25·7	32·1	38·5	45·0	51·4	57·9	64·3
180	6·8	13·6	20·5	27·3	34·1	40·9	47·7	54·6	61·4	68·2
190	7·2	14·4	21·6	28·8	36·0	43·2	50·4	57·6	64·8	72·0
200	7·6	15·1	22·7	30·3	37·8	45·4	53·0	60·6	68·1	75·7
210	7·9	15·9	23·8	31·8	39·7	47·7	55·6	63·6	71·5	79·5

Table (continued)
Total Amount of Alcohol in the Body (in Millilitres)

Weight of Subject (in lb.)	*Per cent Alcohol in the Blood (Grammes per 100 ml.)*									
	0·11	0·12	0·13	0·14	0·15	0·16	0·17	0·18	0·19	0·2
100	41·6	45·0	49·1	52·4	56·8	60·0	64·3	67·4	72·0	75·6
110	45·8	50·0	54·1	58·2	62·5	66·6	70·8	74·8	79·1	83·3
120	50·0	54·4	59·1	63·6	68·2	72·6	77·2	81·8	86·3	90·9
130	54·1	59·0	64·0	68·8	73·8	78·8	83·7	88·6	93·5	98·4
140	58·3	63·6	68·9	74·2	79·5	84·8	90·1	95·4	100·7	106·0
150	62·5	68·2	73·8	79·6	85·2	90·8	96·6	102·2	108·0	113·6
160	66·6	72·6	78·7	84·6	90·9	96·8	103·0	109·0	115·1	121·0
170	70·8	77·0	83·7	90·0	96·6	102·8	109·4	115·8	122·3	128·7
180	75·0	81·8	88·6	95·4	102·2	109·2	115·9	122·8	129·5	136·3
190	79·1	86·4	93·5	100·8	108·0	115·2	122·3	129·6	136·7	144·0
200	83·3	90·8	98·4	106·0	113·6	121·2	128·7	136·2	143·9	151·4
210	87·5	95·4	103·4	111·2	119·3	127·2	135·2	143·0	151·1	159·0

Table (continued)
Total Amount of Alcohol in the Body (in Millilitres)

Weight of Subject (in lb.)	*Per cent Alcohol in the Blood* (*Grammes per* 100 *ml.*)									
	0·21	0·22	0·23	0·24	0·25	0·26	0·27	0·28	0·29	0·3
100	79·5	83·3	87·1	90·9	94·6	98·3	102·2	106·0	109·0	113·5
110	87·5	91·6	95·8	100·0	104·0	108·3	112·5	116·4	120·8	124·8
120	95·4	100·0	104·5	109·0	113·5	118·1	122·7	127·2	131·8	136·3
130	103·4	108·3	113·2	118·0	123·0	128·0	133·0	137·6	142·7	147·6
140	111·3	116·6	122·0	127·2	132·5	137·8	143·1	148·4	153·7	159·0
150	119·3	125·0	130·6	136·4	142·0	147·7	153·4	159·2	164·7	170·4
160	127·2	133·3	139·3	145·4	151·4	157·5	163·6	169·2	175·7	181·6
170	135·2	141·6	148·0	154·5	160·9	167·3	173·8	180·0	186·7	193·0
180	143·1	150·0	156·8	163·6	170·5	177·2	184·0	190·8	197·7	204·5
190	151·1	158·3	165·5	172·7	180·0	187·0	194·2	201·6	208·6	215·7
200	159·0	166·5	174·1	181·8	189·0	196·9	204·5	212·0	219·6	227·1
210	167·0	175·0	182·9	190·8	198·8	206·7	214·7	222·4	230·6	238·6

The Identification of Human Blood Stains

A Critical Survey

* A. C. Hunt, M.D. (Lond.)
† Colin Corby, M.B., B.S. (Lond.)
Barbara E. Dodd, M.Sc. (Lond.), Ph.D. (London)
(*Lecturers*)

and

Francis E. Camps, M.D. (Lond.)
(*Reader*)

From the Department of Forensic Medicine, the London Hospital Medical College, London

The 'short cut' must always be scientifically deprecated. Nevertheless, it affords a temptation on occasions, because of its time- and labour-saving potentiality, which can (unless rigidly disciplined) become a habit. Even more is this important in the forensic sciences, when a consequent error may endanger human life and liberty.

This is exemplified by the examination of material for the presence of human blood stains, identification of which may be a deciding factor in the trial of any case associated with injury or death from violence.

The scientific criteria mentioned below are well recognized,[2, 3, 6, 11, 12] but their establishment in practice may present difficulties as, for example, when only minute traces are present. The problem also entails the consideration of such matters as substances which are alleged to give false positive reactions in the screening test, the age of the stain and the effect on it of exposure to heat or contact with certain substances.

Although visual examination with or without the use of the microscope may be of value, it is not proposed to deal with such examinations.

To prove that a stain is due to human blood, it is first essential to show the presence of haemoglobin and, secondly, that the stain is of human origin. The result of the examination will obviously be of even greater significance if the blood can be shown to be similar to or different from that of a particular individual.

If the evidence is to be maintained at a scientific level, it is highly desirable that these criteria should be observed and that their substitution

* Now Lecturer in Forensic Pathology, University of Bristol.
† Now Lecturer in Forensic Pathology, University of Durham.

by presumptive tests should be avoided. For occasions do occur when a garment which is expected to be contaminated with blood gives a positive presumptive test for blood, although the deposit is insufficient or unsuitable for more specific tests. However tempting it may be to use this as evidence, it is scientifically and morally incorrect to do so, for it is clearly recognized that such tests are not specific and their introduction into evidence may well mislead. It is no argument that the evidence can be challenged, for to do so implies scientific advice which is not always available.

It was, in fact, a case of murder, in which the victim sustained multiple blows on the head, which drew our attention to the importance of discerning between the presumptive tests for blood on the one hand, and the specific tests on the other. The suspect willingly agreed to submit himself to scientific investigation and it seemed most probable that the assailant would have been contaminated by blood splashes or spray in view of the nature and extent of the injuries. For this purpose the benzidine test was employed initially, using filter paper smears from his scalp, body, clothing and property. This last, a leather container, was suggested as having been used for the removal of the weapon which was, in fact, never discovered. Scrapings were also taken from the fingernails.

Some of the material gave a positive benzidine reaction, but was stated to be insufficient to carry the matter any further.

In giving evidence upon the results he had obtained, the scientific witness initially mentioned that the method employed, which was the benzidine reaction, was a presumptive test, but later suggested (in reply to questions) that in specialized hands it could be accepted as specific for blood, an opinion (it was put forward) which would depend upon the speed and intensity of the blue colour produced. It was, as far as can be recollected, never suggested that a positive reaction labelled it as of human origin although it was clearly the impression in court. In reply to a question by the Judge as to exact time required for the colour to appear to indicate that it was blood, the witness was unable to give any figure.

Since textbooks on Forensic Medicine appear to give no clear indication about the relative merits of the various tests for blood stains, it was decided to make a critical experimental survey of those commonly recommended. The results of these observations are here recorded.

Presumptive Tests for Blood

In the investigation of most crimes of violence it is obviously necessary to have some screening test when searching for evidence of blood contamination or spillage.

For this purpose several tests are described which, if positive, afford an indication of the site of material which should be submitted to specific

examination. It can, moreover, be broadly stated that, in experienced hands, a negative result calls for no further examination provided that the reagents are satisfactory and the technique is correct.

Of these presumptive tests the benzidine test is invaluable for the quick exploration of large quantities of material, particularly dark clothing, for possible blood contamination. It thereby assists the investigator in selecting those areas suitable for further examination by specific methods. It cannot be over-emphasized that a positive benzidine reaction, however strong or rapidly obtained or however experienced the investigator, does not *per se* prove scientifically the presence of human blood.

For the purpose of evaluation, 4 series of experiments were carried out:

Series I is a critical survey of some of the presumptive tests for blood in current use.

Series II records a number of parallel tests on blood spots on white cotton cloth measured with a micrometer syringe.

Series III is a further series of parallel tests on unmeasured blood spots on white cotton cloth which are illustrated actual size.

Series IV comprises tests on a cotton cloth finely sprayed with blood.

Description of Experimental Methods

Chemical Methods

1. *Benzidine Test.* To one part of a 10% solution of benzidine in glacial acetic acid, 2 parts of hydrogen peroxide (100 volumes) are added.

A positive reaction is obtained if, on the addition of 1 drop of the extract of a suspected stain, a blue colour immediately appeared.

Alternatively, the stain may also be tested by rubbing the material with filter paper damp with saline and adding 1 drop of the benzidine solution to the paper in order to obtain an immediate blue colour.

A portion of the cloth which is not contaminated must of course be tested as a control.

2. *Orthotolidine Test* (*Kohn and O'Kelly*). The stock solution is prepared by making a 4% solution of orthotolidine in ethanol. If stored at approximately 4° C. it partly precipitates, but this does not affect its efficiency.

The working solution consists of equal parts of stock solution, glacial acetic acid and distilled water mixed together. If this is stored at 4° C. in the refrigerator, it keeps for at least a month.

The test is performed by taking equal volumes of the working solution and 20 volumes of hydrogen peroxide. This mixture was added to the suspected stain or filter paper rubbing, a positive result being indicated by a green or blue colour.

3. *Leuco-Malachite Green Test.* The reagent is prepared according to the following formula:

Leuco-malachite green	1 g.
Glacial acetic acid	100 ml.
Distilled water	150 g.

The reagent is a slightly greenish colour and the stock solution should be kept in a dark brown bottle.

The test is carried out in the same way as the benzidine test, a positive result being obtained by the development of a bright green colour.

4. *Phenolphthalein Test.* 2 g. of phenolphthalein and 20 g. of potassium hydroxide are slowly boiled in 100 ml. of distilled water, adding about 20 g. of powdered zinc during the process. Boiling is continued until the solution becomes colourless and the phenolphthalein thereby reduced to phenolphthalin, which is colourless in alkaline solution. This may take 2–3 hours of boiling and a reflux condenser will have to be used to prevent evaporation. When cool, this solution should be kept in a brown bottle and some of the zinc added to aid in keeping it in a reduced form.

To perform the test a few drops of the stain extract are placed on a watch glass and a drop or two of the phenolphthalein reagent added. When several drops of a solution of hydrogen peroxide are added the appearance of a pink colour shows that the reaction is positive.

5. *Luminol Test.*[8] A solution of 3-aminophthalhydrazide hydrochloride freshly mixed with a source of oxygen such as sodium perborate or hydrogen peroxide in an alkaline solution luminesces strongly in the presence of blood staining.

A solution is made as follows:

3-aminophthalhydrazide	1 g.
Sodium Carbonate	5 g.
Hydrogen Peroxide (10 volumes)	50 ml.
Water to 1 litre.	

The solution is placed in an all-glass atomiser, the garment hung on a suitable clothes hanger or model in a darkened room and the surface sprayed with the atomiseer. Luminous spots are marked and subjected to further investigation.

6. *Haemochromogen Crystal Test (Takayama Method).* The Takayama solution is made up as follows:

10% sodium hydroxide	3 ml.
Pyridine (Analar)	3 ml.
Saturated solution of glucose	3 ml.
Distilled water	7 ml.

The solution is kept in a dark bottle and used for 3 or 4 weeks after being made. Two or three drops are added to a teased-out blood-stained thread of material on a microscopic slide and the preparation enclosed with a cover slip. The slide is then gently warmed to hasten the forma-

tion of the feathery pink crystals which are characteristic of haemochromogen and which can only be formed if the original stain contains blood.

Serological Methods

Preparation of Saline Extracts. Extracts of blood stains for grouping, antihuman globulin and precipitin tests are made with saline. The quantity used varies with the size of the blood spot, ranging from 0.5 to 1.0 ml.

The stained cloth is cut into small pieces and left to soak in saline overnight at 4° C. A piece of unstained material is cut up and soaked in the same amount of saline as a control. In circumstances in which the cloth is thick and entirely soaks up the saline, the extract is recovered by using a centrifugal filter. This device is normally used for the filtration of small quantities of material through a pad to remove bacteria. Without the pad it can easily be adapted for the expression of fluid from pieces of thick cloth, the recovery from cloth 2 inches square treated with 1 ml. of saline being 0.8 ml.

Table 1: Results of ABO Grouping Tests on an Extract of Stained Material with Unstained Cloth as Control.

Antiserum	*Serial Dilutions of Antiserum* 1	2	4	8	16	Titration Value
Original anti-A	V	++	+	W	—	8
Anti-A after mixture with extract from stain	(+)	—				1
Anti-A after mixture with extract from cloth control	V	+	(+)	W	—	8
Original anti-B	V	V	++	(+)	—	8
Anti-B after mixture with extract from stain	V	V	+	+	—	8
Anti-B after mixture with extract from cloth control	V	V	++	(+)	—	8

Key To Symbols

V = Macroscopic agglutination
++ = Very large clumps, few free cells
\+ = Large clumps, more free cells
(+) = Small clumps, free cells
W = Evenly distributed clumps up to 8 cells approx.
— = No agglutination

ABO Grouping by Inhibition Technique. The same anti-A and anti-B sera are used for the whole investigation. These were fairly potent sera diluted just before use so that each gave a titre of 1 : 8 to 1 : 16 with the standard group A_2 and group B cells used.

The saline extract is mixed with the anti-serum. This is effected in one of two different ways, according to the quantity of saline extract of stain available.

The method of choice is to make a 'master titre' of each antiserum running out from this the requisite rows of serially diluted serum for the tests. Then to each tube of a row an equal volume of saline extract is added and the tests left for 2 hours at 4° C.

The control row receives a volume of saline in each tube in place of the extract.

When only a small amount of saline extract is available, an equal volume of this and the appropriate antiserum are mixed and left for 2 hours at 4° C. At the end of this time the mixture is titrated and at the same time a titration of the original serum with a volume of saline added in place of the extract is made.

The 'master titre' method has the advantage that any experimental variation in making the serial dilutions affects all the rows almost equally; therefore the results of titres with and without addition of saline extract of the material being tested are strictly comparable. Moreover, the addition of a volume of extract to a series of tubes containing the antiserum in gradually diminishing strength increases the sensitivity of the test.

After the absorption period at 4°-C., a volume of a 2% suspension of subgroup A_2 cells is added to all tubes containing anti-A and a volume of the same strength group B cells to all tubes containing anti-B. The results are then read after $1\frac{1}{2}$–2 hours' incubation at room temperature.

The presence of the A or B blood group substance in the saline extract of the material is not considered proven unless it can be shown to have brought about a reduction of titre of the original antiserum of more than 2 dilutions. Any smaller reduction than this is considered suggestive but not conclusive. A typical protocol is shown in Table 1 which demonstrates A activity in the extract of the stain under test.

Anti-Human Globulin Inhibition Test (Human Specific). This test detects human globulin. Using saline dilutions of serum it remains positive to a dilution of serum of at least 1 : 10,000. The method does not seem to have been used widely in this country in forensic practice but has been fully described.[10]

The basis of the method is that human globulin will inhibit the activity of anti-human globulin serum (Coombs' reagent), the inhibition being demonstrated by the failure of the anti-human globulin to agglutinate red cells sensitized by incomplete antibodies.

An anti-human globulin is used which when diluted 1 : 400 gives a titre of 1 : 8 against D-positive red cells strongly sensitized by incomplete anti-D. It is mixed with the test fluids by one of the two methods described above for ABO grouping and the mixtures left on the bench for at least 2 hours. A drop of fluid from each tube together with drops from an original titre are tested against the strongly sensitized cells on an opalescent white tile (Fig. 1).

A reduction in titration value of more than 2 dilutions is indicative of the presence of human globulin in the extract of the stain being tested.

Precipitin Test. A few drops of (*a*) saline extract, (*b*) saline extract of

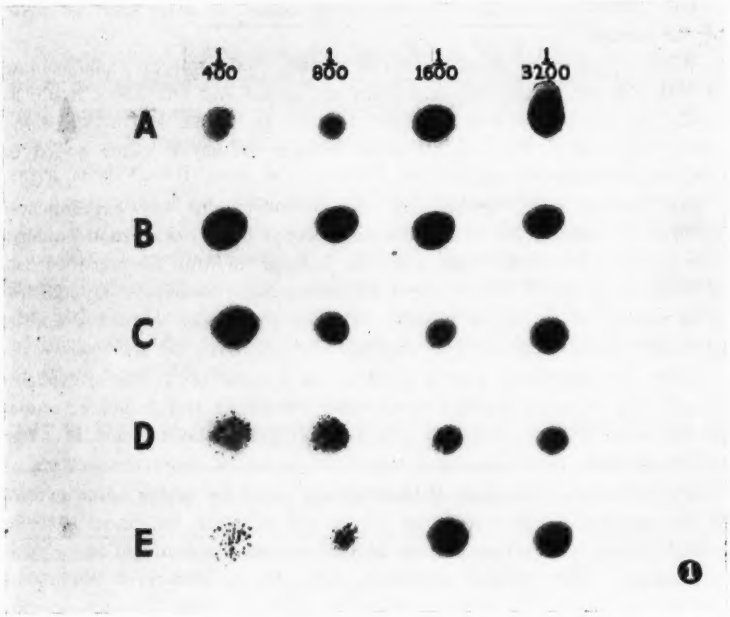


Fig. 1. Results of anti-human globulin inhibition tests on areas of cloth sprayed with blood.

A: Least concentrated area between points marked C and D in Fig. 5.
B: Slightly more concentrated area between B and C in Fig. 5.
C: Area of highest concentration between points marked A and B in Fig. 5.
D: Unstained control.
E: Original titre of anti-human globulin.

unstained cloth as control, (*c*) human serum 1 : 1,000 as positive control, (d) clean saline, are placed separately in 4 precipitin tubes. A small quantity, not accurately measured, of rabbit anti-human precipitating serum supplied by Burroughs-Welcome is carefully run under the fluid already placed in each tube. After 10–20 minutes the tests are read holding the tubes in a good light against a dark background. The development of a white ring caused by precipitation through introduction of antigen in the extract with precipitin in the rabbit serum at the junction of the 2 fluids indicates a positive reaction and shows the presence of human serum in the extract being tested.

When only small quantities of saline extract are available, capillary tubes are used. However, this was avoided whenever possible as in our experience the positive reactions are more difficult to see.

Results

Series I

1. *Benzidine Test.* In the evidence of the case referred to in the introduction, it was alleged that in certain circumstances the benzidine test was specific for blood. These were said to be an appearance of a dark blue colour, an immediate reaction and an experienced operator.

Timing of Reaction. In the following investigations attempts were made to record the time taken for the colour to appear with a stop-watch, although the shortness of the time interval involved makes the observations only approximate.

Solutions and Reagents. The test solutions were made by adding 1 ml. of hydrogen peroxide to 0.2 g. of benzidine in 1 ml. of glacial acetic acid. Attempts were first made to assess the influence of the reagents used on the results of the test. The manufacture of Analar benzidine was discontinued in 1952 owing to the risk of carcinogenesis. British Drug

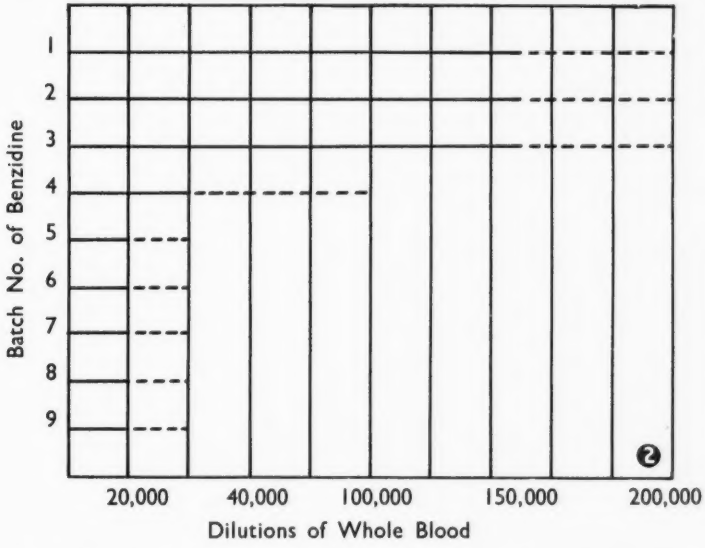


Fig. 2. Comparison of the Efficiency of Different Batches of Benzidine Tested against Dilution of the Blood
The solid lines indicate a strong immediate positive result and the dotted lines a delayed positive result.

Houses, Ltd. supplied us with 3 batches of benzidine, the assay of which showed not less than 98% (via sulphate). Other manufacturers said that they were having increasing difficulty in obtaining benzidine of any degree of purity and efficacy, and it seems likely that this will become general.

Nine different batches of benzidine were tried against known watery dilutions of whole blood, using 100 volumes of peroxide. The dilutions were dried on filter paper and the test solution applied to the dried spot. Fig. 2 shows the results obtained.

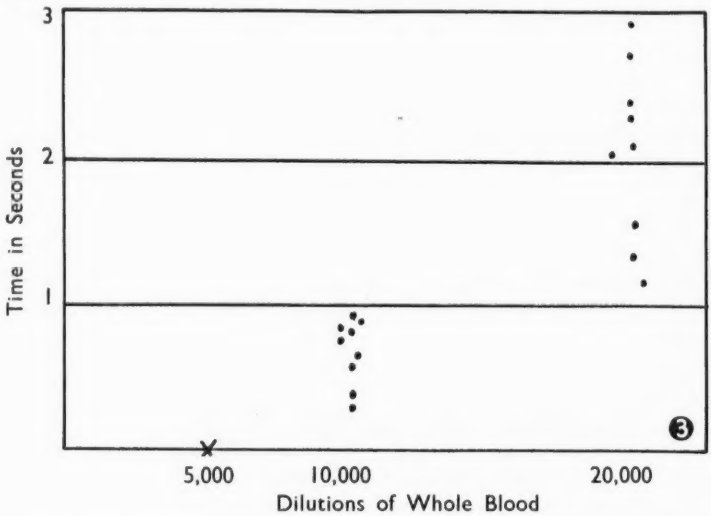


Fig. 3. Time of appearance of colour in the benzidine test compared with dilutions of whole blood.

The more diluted the blood the less intense was the colour reaction and the longer it took to appear (Fig. 3). Considerable variation was noted in the colour produced by different batches with the weaker blood dilutions, and one batch gave a distinctly green-blue appearance.

The strength of the hydrogen peroxide solution used also made a noticeable difference to the strength of the colour and its speed of reaction. Hydrogen peroxide (20 volumes) approximately doubled the time taken in dilutions above 1 : 5,000. For all further tests one of the more sensitive batches of benzidine and hydrogen peroxide (100 volumes) was used.

Tests Applied to Blood Stains

Experiments were next made on human blood stains.

The size of blood spots made by hitting a pool of blood with a hammer were roughly estimated; 10 ml. of blood were placed on a tiled floor,

and struck hard with a domestic hammer. Glass microscope slides were arranged around this at varying distances, and the resulting splashes measured microscopically with an eye-piece micrometer. At a distance of up to 6 inches there were, in addition to large splashes, numerous fine flecks between 40 and 100 microns in diameter which were just visible to the naked eye. At 12 inches there were no drops larger than 20 microns in diameter, and at 2 feet there was none larger than 0.5 micron. The smallest drop measured with a micrometer syringe when produced on to cloth was of the order of 0.0001 millimetre (i.e. approximately the size of the smallest drops found at 2 feet). Drops of this size (0.0001 millimetre) were put on to 2-inch squares of different types of cloth and allowed to dry for 24 hours. The whole 2 inches were then rubbed lightly with filter paper, which was tested with benzidine solution.

Tests Applied to Other Substances than Blood

Besides the tests on human blood, others were made on substances alleged to give a false positive reaction with benzidine. These can be divided into chemical oxidizing agents and biological material.

Chemical Oxidizing Agents: These, such as potassium permanganate, potassium ferrocyanide and 'Steroxol,' give an immediate dark blue colour with benzidine reagent at very high dilution comparable to that of blood. However, when even a strong solution was allowed to dry on cloth, none could be transferred to dry filter paper. The stain itself, as might be expected, gave a strong colour if the reagent was applied to the cloth, and small amounts were capable of being transferred to the filter paper if damp.

Human Faeces: Specimens of normal human faeces frequently produced a positive reaction, a few giving a strong immediate dark blue colour, but in most cases in 2 or 3 seconds.

Vegetable Material: Various vegetable materials were also tested. Green leaves all gave a positive benzidine reaction when smeared on filter paper, the colour appearing in 1.5 to 4.5 seconds. When a distinct green stain had been transferred to the paper, the colour was occasionally as intense as that given by blood. Paper wiping of green vegetable stains on cloth only gave a positive benzidine test if visible vegetable fragments were transferred, the time of appearance being between 2 and 5 seconds.

Juice from potatoes and tomatoes gave a strong immediate positive when wet, but only a faint positive when dry, and when dried on cloth could not be transferred to filter paper. No positive reaction was seen with juice from apples, oranges, grapes, pears or carrots.

Leather and Shoe Polish: Ten different types of *new leather* were tested. Wipings from 2 of these showed very small flecks of reaction in 1-2 seconds. 'Ox blood' polishes were examined, and none gave a positive benzidine reaction.

A random sample of 30 used shoes from a repair shop was tested. A strong positive reaction which appeared in 3 seconds was obtained from one, and a weak positive reaction (from 5 to 10 seconds) was given by a further 6.

Random Samples of Coats and Clothing: The fronts of the jackets of 15 men in a public house were wiped with filter paper. The results were negative apart from a few flecks appearing after 5 to 10 seconds.

The overalls of *2 greengrocers* were rubbed with filter paper and a few pale flecks and streaks appeared after 5 seconds. Finger nail scrapings from these greengrocers were strongly positive in 1 to 2 seconds.

Human Skin and Hair: Wipings were taken from the axillae of 10 normal men. Strong immediate dark blue reactions were given by 3. A few streaks and flecks of colour appeared in 2 to 3 seconds on filter papers rubbed on the front of the chests of these 10 men. Rubbings of head hair were all negative.

A considerable number of other substances was examined with negative results.

Orthotolidine Test: Using the strength recommended by Kohn and O'Kelly[7] for occult blood, the orthotolidine test seemed to give identical results to the benzidine test. In view of the fact that benzidine with any degree of purity will become difficult to obtain, this test may possibly replace the benzidine test.

It gave positive results on all the spots and sprays examined as well as a weak, delayed positive result on watery dilutions of blood up to 1 : 1,000,000.

Similar false positives were obtained with chemical oxidizing agents, formalin, green leaves (particularly cauliflower), iron salts and iodides in the same way as the benzidine test.

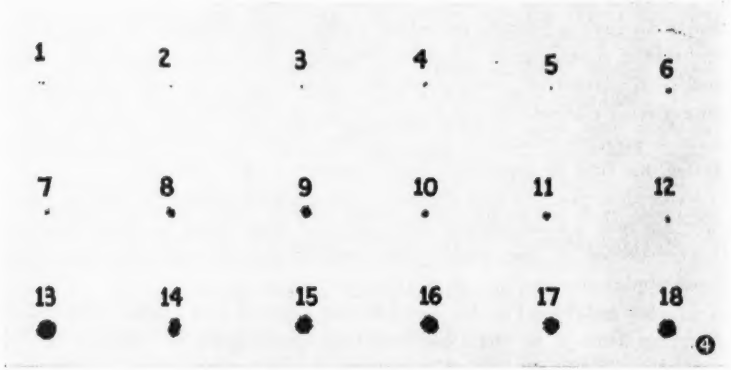


Fig. 4. Blood spots used for tests in Series III, shown life size.

Malachite Green Test: The larger spots of blood (Fig. 4) and the heavily stained area of the spray (Fig. 5) gave a positive result when the malachite green test was applied direct to the stain. Filter paper rubbings over the surface of these stains, however, failed to give a positive result when the benzidine was strongly positive.

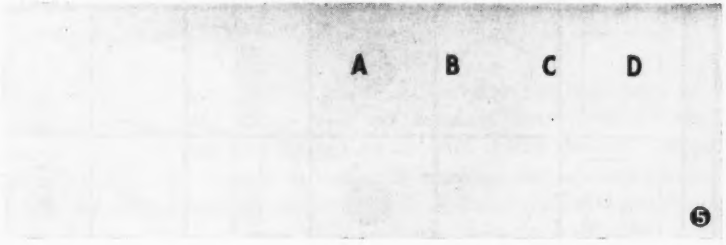


Fig. 5. Cloth finely sprayed with blood, almost completely invisible to the naked eye.
The areas marked A, B, C, D, were used in the experiments in Series IV.

We found the malachite green test to be more specific than benzidine, only giving delayed weak positives with potatoes and tomato juice when wet and a negative result to milk and formalin. A strong positive result was obtained with oxidizing agents such as potassium permanganate.

Trouser leg rubbings from a pathologist actively engaged in post-mortem work, and from a mortuary table gave negative results with malachite green, whereas a strong immediate reaction was obtained with benzidine.

It was concluded that the apparent increase of specificity of the malachite green test was, in fact, due to its diminished sensitivity and that it was not as useful in screening smaller and finer blood stains as the benzidine test.

Luminol Test: Although this test was found to give positive results with all the spots and sprays examined, direct application to the stain itself tended to harm it for further examination. Filter paper rubbed over the spots and sprayed areas and then tested with Luminol were all negative.

Phenolphthalein Test: This was found to be very sensitive for blood, giving a positive reaction at dilutions of 1 : 10,000,000. It was found, however, only to react satisfactorily with extracts of blood stains and not directly with the smaller stains or on filter paper rubbings.

It also appeared to be more specific for blood, giving only false positive results with copper salts.[4]

Series II

Parallel Tests on Blood Spots Measured with a Micrometer Syringe. (*a*) Benzidine, (*b*) anti-human globulin, (*c*) precipitin and (*d*) ABO grouping

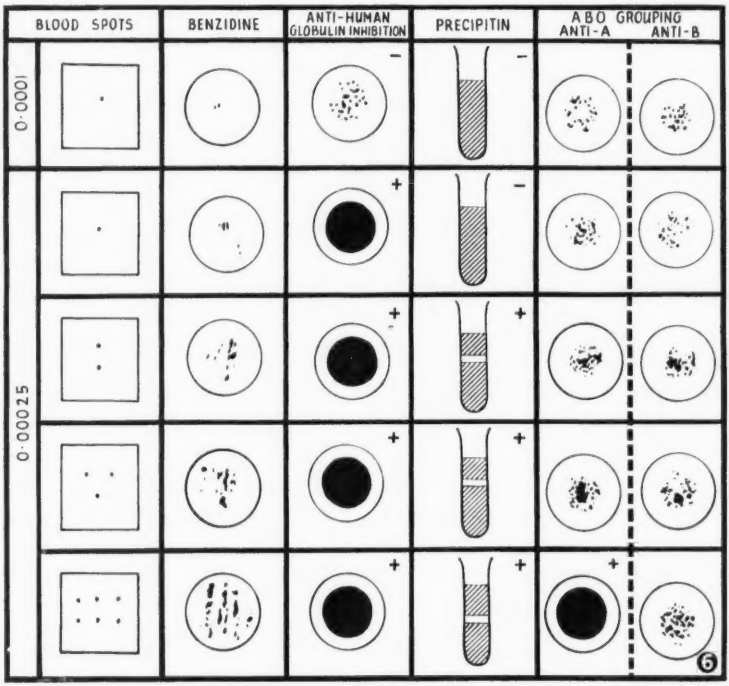


Fig. 6. Diagrammatic representation of the results of the parallel tests in Series II.

carried out in parallel on blood spots ranging from 0.0001–0.0015 ml. are shown in Fig. 6.

The anti-human globulin test was positive in all cases except the single spot of 0.0001 millimetre. In later experiments, however, with an improved technique, spots of this order gave good positive results.

Table 2: Saliva and Tears Tested by Anti-human Globulin Inhibition Test.

	Serial Dilutions of Anti-human Globulin			
	400	800	1600	3200
Original Titre of A.H.G.	4+	4+	2+	—
After mixture with:—				
1. Saliva 1 in 2	—	—	—	—
1 in 10	4+	3+	—	—
1 in 50	4	3+	—	—
1 in 100	4+	4+	3+	—
2. Tears 1 in 2	—	—	—	—
1 in 5	—	—	—	—
1 in 50	3+	3+	—	—
1 in 100	4+	4+	2+	—

On the other hand, approximately 0.0008 ml. blood was required for a positive result from the precipitin test and as much as 0.001 ml. was necessary before conclusive grouping results were obtained.

Although haemochromogen crysal tests were not performed in this series, later observations showed that a positive result could be obtained when only about 0.0002 ml. of blood could be devoted to this test.

Series III

A series of blood spots roughly graded according to size were made on a piece of white cloth. These were not measured but are shown actual size in Fig. 4. In each case the amount of blood in each spot was sufficient to soak through the cloth to the back. A benzidine test performed according to the method described above was positive for all the blood spots made.

A certain number of spots from each row were then subjected to the following tests:

(*a*) Haemochromogen crystal;
(*b*) Precipitin;
(*c*) Anti-human globulin;
(*d*) ABO grouping.

Selected results are given in Table 3.

Table 3: Results of Tests on Blood Spots Nos. 6, 8 and 13, Shown in Fig 4

	Tests Performed			
	Haemochromogen	*Precipitin*	*Anti-human Globulin*	*ABO Grouping*
No. 6	+	+	+	—
No. 8	+	+	+	—
No. 13	+	+	+	+

It will be seen that very small spots can be shown to be human blood, but something of much larger size is required before an ABO grouping result can be considered conclusive.

Series IV

The final experiment was to spray a piece of cloth as finely as possible with blood. To achieve a fine spray, a compressed air pump was used. The result can be seen in Fig. 5. The area of highest concentration is between the points marked A and B and the least, between the points marked C and D.

Tests were made on 3 areas each of 1 sq. cm.

(*a*) This was from the area so lightly sprayed that the blood spots were almost invisible, even on white cloth.
(*b*) This was from a slightly more concentrated area.
(*c*) This was from the point of highest concentration of blood.

From a knowledge of the approximate quantity of blood sprayed over the whole cloth, it was calculated that, given an even distribution, the amount of blood per 1 sq. cm. was of the order of 0.0001 ml.

Haemochromogen tests were performed first and were negative in all cases. Saline extracts were then made by soaking each piece of cloth in 0.5 ml. of saline overnight. A piece of unstained cloth of the same material was included as a control.

The ABO grouping and anti-human globulin inhibition tests were carried out using the 'master titre' method of titration. The blood used in the experiment was known to be of group A but the group could not be established in any of the pieces tested. On the other hand, the anti-globulin inhibition test was positive on (*a*), (*b*) and (*c*), but not on (*d*), the unstained control. These results are shown in Fig. 1. The precipitin test was positive on (*b*) and (*c*), but negative on (*a*) and (*d*).

Thus the presence of human material could be demonstrated, but not that it was, in fact, blood.

Discussion of Results

A presumptive test for blood is of great value as a screening operation, especially as all blood stains are not by any means always recognizable to the eye, especially when masked by the colour of a dark cloth material or when thinly spread (Fig. 5). Further, those areas which give the strongest reactions in the presumptive test indicate the most promising site for further haematological and serological tests.

Of the 4 chemical tests investigated, the benzidine test or the ortho-tolidine test was found to be the most satisfactory because of the technical simplicity (filter paper), the ease of obtaining definite positive results and the fact that no harm is sustained by the stain itself. The disadvantage of the non-specificity is not considered important when used as a preliminary screening test to decide whether the stain under investigation *may* be blood, or when negative to exclude blood.

The malachite green test was similar to the benzidine test, but far less sensitive, thus possibly giving the false impression of being more specific.

The Luminol test may be of value in examining large areas, but has the disadvantage of requiring a dark room and also makes part of the stain area useless for further examination.

Finally, although the phenolphthalein test seems to be very specific and sensitive for blood, the reagents are tedious to prepare and it was found only to be satisfactory on extracts of blood stains. When applied direct to the smaller spots or on filter paper rubbings it failed to give a positive result.

Scientific Proof of Presence of Human Blood

This can only be postulated when a test for *haemoglobin* and a test for *human protein* are both found positive.

The most reliable method for demonstrating haemoglobin appears to be the haemochromogen crystal test. It is, moreover, easy to perform and gives positive results with a small amount of material.

The choice between precipitin and antiglobulin inhibition tests may well be influenced by personal preference. The experience of one of the authors (Barbara Dodd) is that the anti-human globulin test proves to be slightly more sensitive and is easier to read although it takes longer to perform. Moreover, it is not dependent upon the law of optimum proportions, as is the precipitin reaction. (If the extract of a blood stain contains too high a concentration of protein the 'ring' will not appear). In addition, the saline extract of a stain does not have to be perfectly clear, as is essential for the precipitin test. It is difficult to agree with Glaister[5] that dependence on a negative result of inhibition of agglutination detracts from its reliable application in legal issues. In fact, this dependence upon the presence of a substance being revealed by its power to inhibit an expected reaction is shared by the method of detecting A and B antigens in blood stains, which is also an inhibition technique.

Both the anti-human globulin test and the precipitin test give positive results with blood of some of the higher primates.[10, 13] Therefore a statement that a stain is of human origin based on the tests as described above must be accompanied by this qualification. In most instances cross-reaction with the blood of higher apes will not affect the case, but in some countries, e.g. South Africa, this cross-reaction may present a problem. In the solution of this problem, the work of Boyden *et al.*[1] (using the Libby Phetronreflectometer) may be of great value.

The anti-human globulin test unaccompanied by a test for haemoglobin cannot be considered specific for blood, since other body fluids besides blood contain globulin, e.g. saliva. Results of tests on other body secretions are shown in Table 2. However, it is clear from this Table and from Fig. 6 that blood gives a positive reaction at a far higher dilution than does other human material. This fact may help to elucidate the origin of unknown material being tested.

As the above results show, the ABO group of a stain was more difficult to establish than the fact that it was of human blood. This difficulty was almost certainly because the tests were limited to very small quantities of material. Moreover, the saline extract of the stain quite obviously did not contain all the blood which formed the stain and, in addition, a small quantity of stain had to be reserved for the haemochromogen crystal test. Provided control tests on unstained cloth are satisfactory, more

definite results may be obtained by adding the suitably diluted antiserum directly on to the cloth without first making a saline extract. This was carried out with advantage on some of the spots examined in this investigation. A further variation of technique with the object of conserving material is the addition of the anti-A and anti-B sera as a mixture to the stain or to a saline extract. Thus only half the quantity of material being tested is required.

It cannot be over-emphasized that only a large reduction in titration value may be taken as evidence of the presence of a blood group substance, particularly as a test for the presence of agglutinins (which is an essential routine in the testing of liquid blood) usually has to be omitted unless the stain is large and relatively fresh.

While a positive result, if definite enough, may prove the presence of a blood group antigen, a negative result cannot be taken as indicative of its absence from the blood which caused the particular stain. Thus the conclusion that blood is of group O can never be based merely on the finding that it gives no inhibition of the reactivity of anti-A and anti-B. An inhibition test with anti-H may yield helpful results. A reduction in titre of anti-H but not of anti-A or anti-B would strongly suggest that the stain was from a group O individual, particularly since H activity diminishes more quickly than A and B.

Positive results for the presence of agglutinins in the stains are, of course, of value in confirming the true absence of an agglutinogen. The limits imposed by the above experiments did not allow for tests for agglutinins or for H.

It is difficult to establish any age limit beyond which a stain is likely to be too old for reliable testing. There is a wide variation due to the conditions to which it has been exposed before testing. In most of the experiments the blood was tested within a few days of being shed, although repetitions of tests after a few months yielded results which did not appreciably differ from those on the fresher specimens. It may be of interest to report that examination of blood crust which had remained on a piece of wood for nearly 10 years (R. *vs.* Hume, Central Criminal Court, 1950) and for most of its life had formed part of a museum specimen within a glass case, gave a positive haemochromogen test, although the crystals formed more slowly than usual. The anti-human globulin test was negative even after prolongation of the absorption stage of the test as suggested by Ruffié and Ducos;[10] but there was evidence of the presence of the blood group substance A. Normally this grouping result would be interpreted with caution since an accumulation of dust on a stain through the passage of time might be the cause of an 'A reaction.' The specimen was protected by glass and was therefore almost certainly free from extraneous matter.

Substances similar to A, B and H were demonstrated some years ago (Rimington *et al.*[9]) in crude extracts of house dust. These caused quite large inhibitions of anti-A and anti-B antibodies.

Collected laboratory dust was found to give complete inhibition of anti-A although not of anti-B.

Summary

1. The danger of acceptance of presumptive tests for blood as proof of specificity is stressed.

2. The various accepted chemical and serological tests for identifying blood are outlined.

3. A summary of the presumptive tests with experimental data is made.

4. Parallel tests are described using blood spots measured with a micrometer syringe.

5. Similar tests are also described using unmeasured spots.

6. Tests carried out on cloth finely sprayed with blood are demonstrated.

7. Various difficulties and the problem of substances giving false positives are discussed.

8. The results of the survey are discussed and the following conclusions are reached:

1. That a presumptive test for blood is of great value for screening with a view to subsequent confirmation and for this purpose the benzidine test or the orthotolidine test appear to be the most satisfactory.

2. Such a presumptive test is not scientific proof of the presence of blood. In order to establish this, a positive test must be obtained first for haemoglobin and, secondly, for human protein.

3. Upon this basis the procedure in the examination of suspected blood stains should consist of:

(*a*) A positive presumptive test;

(*b*) A test for haemoglobin (haemochromogen test);

(*c*) A test to show human protein (anti-human globulin inhibition test);

(*d*) If adequate and suitable material is available, group specific tests may be attempted.

References

1. Boyden, A., Gemeroy, D. and Defalco, R. (1956): The Serological Museum, Bulletin 16, Rutgers University, New Jersey.
2. Camps, F. E. and Purchase, W. B. (1956): *Practical Forensic Medicine*. London: Medical Publications.
3. Gonzales, T. A., Vance, M., Helpern, M. and Umberger, C. J. (1954): *Legal Medicine, Pathology and Toxicology*, 2nd ed. New York: Appleton Century.
4. Glaister, J. (1926): Brit. Med. J., **1**, 650.
5. Glaister, J. (1958): *Medical Jurisprudence and Toxicology*. Edinburgh and London: E. & S. Livingstone Ltd.

6. Kerr, D. (1957): *Forensic Medicine.* Edinburgh: Adam and Charles Black.
7. Kohn, J. and O'Kelly, T. (1955): J. Clin. Path., **8**, 249.
8. McGraph, J. (1942): Brit. Med. J., **2**, 156.
9. Rimington, C., Stillwell, D. E. and Maunsell, K. (1947): Brit. J. Exp. Path., **28**, 22.
10. Ruffié, J. and Ducos, J. (1956): Ann. Med. Leg., **36**, 17.
11. Smith, Sydney and Fiddes, F. S. (1955): *Forensic Medicine.* London: J. & A. Churchill Ltd.
12. Smith, Sydney and Simpson, Keith (1956): *Taylor's Principles and Practice of Medical Jurisprudence,* 10th ed., Vol. 1. London: J. & A. Churchill Ltd.
13. Taylor, J. E. D. (1952): S. Afr. Med. J., **26**, 81.

The Law-Medicine Center

An Experience in Working Together

The philosophy of the Law-Medicine Center in Cleveland is based on the belief that society must solve certain medico-legal problems of man through unity and not diversity, through one center and not two schools, through mutual co-operation and not divisive controversy. Medicine and law, when they have met in the administration of justice, have too often generated much conflict and confusion. Not only have law and medicine suffered, but also justice.

The specific purpose of the Center is to improve the administration of justice (both civil and criminal) through better use of medicine as a source of truth for the law.

In 1953, to achieve this purpose, Western Reserve University and the Cuyahoga County Coroner's Office cooperated to present an instructional program in law-medicine problems. That year the Coroner's Office opened its new $800,000 laboratories on the University campus adjacent to the School of Law, School of Medicine and Institute of Pathology. This public health office of local government has major medico-legal problems in its daily activity. Dr. Samuel R. Gerber, Coroner since 1936, University officials and public-spirited citizens labored for several years prior to educate the voters of the County on the wisdom of voting for the necessary public bond issue to build a new scientific coroner's office in the University environment. By an overwhelming vote the concept and the building were approved.

The co-operation between the governmental office supported by the taxpayers of the county and the privately endowed, voluntarily supported University has grown broader and deeper since 1953. The spearhead for this co-operation is the Law-Medicine Center officially created by the University trustees in 1954.

Administration of the Center rests in the Director, a full-time member of the School of Law Faculty, the Cuyahoga County Coroner, Dr. Gerber, and the Director of the Institute of Pathology, Dr. Alan R. Moritz, who also serves as Professor of Pathology in the School of Medicine and is an internationally recognized leader in Forensic Medicine.

In the University structure, the Center represents not a school but a service institution. For University administration the Center is placed under the Dean of the School of Law. The vigorous support of Dean Emeritus Fletcher Reed Andrews and the present Dean, Dr. Edgar I. King, have been potent factors for success.

The Law-Medicine program completing an operation of 7 years in February 1960, has conducted 52 separate courses or schools for instruction. In civil justice, 16 courses or seminars have been given; in criminal justice, 36 courses or schools. The student registration for civil justice instruction has totalled nearly 1,200 while criminal justice has attracted over 1,300. These students represent doctors and lawyers, law students and medical students, law enforcement officials (police, prosecutors, probation and parole officers, coroners and medical examiners), insurance claimsmen, industrial relations personnel, labor union officials, social workers and others. Nearly 300 individuals have served as instructors both from the University faculty and the community of Greater Cleveland, the state of Ohio and beyond.

Current budget of the Center is $40,000 annually. The bulk of the expense is measured by faculty honoraria. In 1954 a $20,000 grant from the Cleveland Foundation, America's pioneer community trust, enabled the program to begin. Since then several interested corporations and individuals have given a total of $7,500 but in the past year only $1,000 of the income came from gifts. The remaining $39,000 represented tuition and service fees, primarily the former.

The Center has published several books and monographs, has conducted research, has provided surveys for law enforcement agencies and has tested law enforcement personnel. Details of these functions—instruction, publication, research, survey, testing—will be described in articles to appear in *Minnesota Medicine*. Some challenging aspects of future law-medicine problems will also be presented.

The central theme of philosophy, purpose and function at the Center—*to use medicine in law to achieve justice*—is vitally affected by man's current law-medicine knowledge and skills being in a state of volatile change. Law and medicine, when co-operating in the administration of justice, must be prepared to recognize this condition of swift change in modern man's intellect and above all to adapt to this condition to resolve human problems in the medico-legal area. A free, democratic society laboring co-operatively through public offices and private enterprises is our best hope in to-day's challenging world. The Law-Medicine Center is a unique co-operative activity of such labors by government and university to solve a common problem. By-products of the activity are the dignifying of the professions of law and medicine but, more important, better service to the client-patient—an individual human being with complex medico-legal problems.

[Oliver Schroeder, Jr. in *Minnesota Medicine*, 1959, **42**, 1636.]